

EPA/8190  
22.07.2004

REC'D 02 SEP 2004  
WIPO PCT

PA 1168263

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

May 12, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/488,799

FILING DATE: July 22, 2003

PRIORITY  
DOCUMENT

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS



T. LAWRENCE  
Certifying Officer

PATENT APPLICATION SERIAL NO. \_\_\_\_\_

U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE  
FEE RECORD SHEET

07/23/2003 NDAMTE1 00000078 60488799  
01 FC:2005 80.00 0P

PTO-1556  
(5/87)

# Provisional Application For Patent Cover Sheet

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Express Mail Label No.

## INVENTOR(S)

Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Martin F.	Bachmann	Seuzach, Switzerland
Vania	Manolova	Zurich, Switzerland
Tazio	Storni	Viganello, Switzerland

## TITLE OF THE INVENTION (500 Characters Maximum)

Enhancement of A-Type CpG-Induced IFNa-Production by Liposomes: Method of Preparation and Use

## CORRESPONDENCE ADDRESS

*Direct all correspondence to:*

Customer number 26111

Type Customer Number here

OR

<input type="checkbox"/> Firm or Individual Name	STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.				
Address					
Address					
City		State		ZIP	
Country	USA	Telephone		Fax	

## ENCLOSED APPLICATION PARTS (check all that apply)

Specification Number of pages 32

CD(s), Number \_\_\_\_\_

Drawing(s) Number of sheets: 8

Other (specify) Paper copy of Sequence Listing

Application Data Sheet. See 37 CFR 1.76

## METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

Applicant claims small entity status. See 37 CFR 1.27.

A check or money order is enclosed to cover the filing fees

The Commissioner is hereby authorized to charge filing fees or credit any overpayments to Deposit Account Number: 19-0036.

Payment by credit card. Form PTO-2038 is attached.

Filing Fee  
Amount (\$)

80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No

Yes, the name of the U.S. Government agency and the Government contract number are:

[Page 1 of 2]

Respectfully submitted,

Signature: Peter A. Jackman

Typed or Printed Name: Peter A. Jackman

Telephone: 202-371-2600

Date: July 22, 2003

Registration No. 45,986 (if appropriate)

Docket Number: 1700.0450000

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you are required to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

07/29/03  
U.S. PTO  
1821

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# FEE TRANSMITTAL for FY 2003

Patent fees are subject to annual revision.

 Applicant claims small entity status. See 37 CFR 1.27**TOTAL AMOUNT OF PAYMENT (\$)** 80.00

Complete if Known	
Application Number	To Be Assigned
Filing Date	July 22, 2003
First Named Inventor	Martin F. Bachmann
Examiner Name	N/A
Art Unit	N/A
Attorney Docket No.	1700.0450000/JAG/PAJ/TAC

**METHOD OF PAYMENT (check all that apply)**

Check  Credit card  Money Order  Other\*\*  None  
\*\* Charge any deficiencies or credit any overpayments in the fees or fee calculations of Parts 1, 2 and 3 below to Deposit Account No. 19-0036.

Deposit Account  
Deposit Account Number 19-0036

Deposit Account Name: Sterne, Kessler, Goldstein &amp; Fox P.L.L.C.

**The Commissioner is authorized to: (check all that apply)**

Charge fee(s) indicated below  Credit any over payments  
 Charge any additional fee(s) during the pendency of this application  
 Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

**FEE CALCULATION****1. BASIC FILING FEE**

Large Entity Small Entity

Fee Code (\$)	Fee	Fee Code (\$)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee
1002	330	2002	165	Design filing fee
1003	520	2003	260	Plant filing fee
1004	750	2004	375	Reissue filing fee
1105	160	2005	80	Provisional filing fee 80.00

**SUBTOTAL (1) (\$)** 80.00**2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE**

Total Claims	- 20** =	X	=	Extra Fee from below	Fee Paid
Indep. Claims	- 3** =	X	=		
Multiple Dependent			=		

Large Entity	Small Entity	Fee Description
Fee Code (\$)	Fee Code (\$)	
1202 18	2202 9	Claims in excess of 20
1201 84	2201 42	Independent claims in excess of 3
1203 280	2203 140	Multiple dependent claim, if not paid
1204 84	2204 42	**Reissue independent claims over original patent
1205 18	2205 9	**Reissue claims in excess of 20 and over original patent

**SUBTOTAL (2) (\$)** 0.00

\*\*or number previously paid, if greater; For Reissue, see above

**FEE CALCULATION (continued)****3. ADDITIONAL FEES**

Large Entity Small Entity

Fee Code (\$)	Fee (\$)	Fee Code (\$)	Fee (\$)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1502	50	2052	25	Surcharge-late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for ex parte reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1808	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) \_\_\_\_\_

\* Reduced by Basic Filing Fee Paid

**SUBTOTAL (3) (\$)** 0.00**SUBMITTED BY**

Complete if applicable

Name (Print/Type)	Peter A. Jackman	Registration No. (Attorney/Agent)	45,986	Telephone	202-371-2600
Signature				Date	7/22/03

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, O.P. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-788-9199) and select option 2.



ATTORNEYS AT LAW

Robert Greene Sterne  
Edward J. Kessler  
Jorge A. Grindstein  
David K.S. Cumwell  
Robert W. Esmond  
Terry-Genie G. Durkin  
Michaela A. Cimberla  
Michael B. Ray  
Robert E. Sozal  
Eric K. Steffens  
Michael Q. Lee  
Steven R. Ludwig  
John M. Covert  
Linda E. Alcom  
Robert C. Milhonig  
Lawrence B. Bugalsky  
Donald J. Featherstone  
Michael V. Messinger

Judith U. Kim  
Timothy J. Shea, Jr.  
Patrick E. Garret  
Heidi L. Kaus  
Edward W. Yee  
Albert L. Perro\*  
Donald R. Banowitz  
Peter A. Jackman  
Molly A. McCall  
Teresa U. Medler  
Jeffrey S. Weaver  
Kendrick P. Patterson  
Vincent L. Capuano  
Albert J. Rasulo II\*  
Edora Ellison Floyd  
Thomas C. Flala  
Brian J. Del Guono  
Virgil Lee Beaston\*

Kimberly N. Reddick  
Theodore A. Wood  
Elizabeth J. Haanes  
Bruce E. Chalker  
Joseph S. Ostroff  
Frank R. Cunningham  
Christine M. Uhler  
Rae Lynn Prengaman  
Jane Shershenovit\*  
Lawrence J. Carroll\*  
George S. Bardmessner  
Daniel A. Klein\*  
Rodney G. Maze  
Jason D. Eisenberg  
Michael A. Specht\*  
Andrea I. Kamage  
Tracy L. Muller  
Jon E. Wright\*

LuAnne M. Yunock\*  
Registered Patent Agents\*  
Karen R. Markowicz  
Nancy J. Leith  
Ann E. Summerfield  
Helene C. Carlson  
Gaby L. Longsworth  
Matthew J. Dowd  
Aaron L. Schwartz  
Angeline G. Uy  
Mary B. Tung  
Kathina Y. Pei  
Bryan L. Stetton  
Robert A. Schwartzman  
John I. Figueira  
Timothy A. Doyle  
Jennifer R. Mahalingappa

Teresa A. Colella  
Jeffrey S. Lundgren  
Victoria S. Rutherford  
Of Counsel  
Kenneth C. Bass III  
Lisa A. Dunner  
Evan R. Smith

\*Admitted only in Maryland  
•Admitted only in Virginia  
•Practice limited to  
Federal Agencies

July 22, 2003

WRITER'S DIRECT NUMBER:  
(202) 772-8582

INTERNET ADDRESS:  
PJACKMAN@SKGF.COM

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Mail Stop Provisional Application

Re: U.S. Provisional Patent Application  
Appl. No. (*To Be Assigned*); Filed: HERewith  
For: Enhancement of A-Type CpG-Induced IFN $\alpha$ -Production by  
Liposomes: Method of Preparation and Use  
Inventors: Bachmann *et al.*  
Your Ref: PA047USprov  
Our Ref: 1700.0450000/JAG/PAJ/TAC

Sir:

The following documents are being submitted under 37 C.F.R. § 1.53(c) herewith for appropriate action by the U.S. Patent and Trademark Office:

1. PTO Fee Transmittal (Form PTO/SB/17);
2. U.S. Provisional Patent Application entitled:

**Enhancement of A-Type CpG-Induced IFN $\alpha$ -Production by Liposomes:  
Method of Preparation and Use**

and naming as inventors:

Martin F. Bachmann  
Vania Manolova  
Tazio Storni

the application consisting of:

- a. A Provisional Application for Patent Cover Sheet;

Commissioner for Patents

July 22, 2003

Page 2

- b. an Application Data Sheet (37 C.F.R. § 1.76);
- c. A specification containing 32 total pages:
  - (i) 26 pages of description prior to any claims;
  - (ii) 5 pages of claims (27 claims);
  - (iii) a one page abstract;
- d. 8 sheets of drawings: (Figures 1, 2, 3, 4, 5, 6, 7A-7B and 8A-8B);
- e. 4 pages of a paper copy of a sequence listing after the abstract;

3. Authorization to Treat a Reply As Incorporating An Extension of Time Under 37 C.F.R. § 1.136(a)(3);

4. Form PTO-2038 Credit Card Payment Form in the amount of \$80.00 to cover the filing fee; and

5. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and returned as soon as possible.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Peter A. Jackman  
Attorney for Applicants  
Registration No. 45,986

JAG/PAJ/TAC:dms

Enclosures

SKGF\_DC1:158208.1

## INVENTOR INFORMATION

Inventor One Given Name:: Martin F  
Family Name:: Bachmann  
Postal Address Line One:: Goldackerweg 8  
City:: CH-8472 Seuzach  
Country:: Switzerland  
Citizenship Country:: Switzerland  
Inventor Two Given Name:: Vania  
Family Name:: Manolova  
Postal Address Line One:: Pestalozzistr. 41  
City:: CH-8032 Zurich  
Country:: Switzerland  
Citizenship Country:: Bulgaria  
Inventor Three Given Name:: Tazio  
Family Name:: Storni  
Postal Address Line One:: Via Aldesago 10  
City:: CH-6962 Viganello  
Country:: Switzerland  
Citizenship Country:: Switzerland

## CORRESPONDENCE INFORMATION

Correspondence Customer Number:: 26111

## APPLICATION INFORMATION

Title Line One:: Enhancement of A-Type CpG-Induced  
Title Line Two:: IFNalpha-Production by Liposomes: Method  
Title Line Three:: of Preparation and Use  
Total Drawing Sheets:: 8  
Formal Drawings?:: No  
Application Type:: Provisional  
Docket Number:: 1700.0450000  
Secrecy Order in Parent Appl.?:: No

## REPRESENTATIVE INFORMATION

Representative Customer Number:: 26111

Source:: PrintEFS Version 1.0.1

**Enhancement of A-Type CpG-Induced IFN $\alpha$ -Production by Liposomes:  
Method of Preparation and Use**

**Inventors:**    Martin F. Bachmann  
                    Vania Manolova  
                    Tazio Storni

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

[0001]       The present invention is related to the fields of vaccinology, immunology and medicine. Using liposomes, the invention provides compositions and methods for enhancing production of IFN $\alpha$  by DNA, preferably immunostimulatory nucleic acids, and even more preferably oligonucleotides containing at least one non-methylated CpG sequence. Preferred liposomes are cationic liposomes. The invention can be used to induce IFN $\alpha$  in vivo, particularly useful for the treatment of chronic viral diseases, cancer and short-term prophylaxis from pathogen-infection.

**Related Art**

[0002]       The essence of the immune system is built on two separate foundation pillars: one is specific or adaptive immunity which is characterized by relatively slow response-kinetics and the ability to remember; the other is non-specific or innate immunity exhibiting rapid response-kinetics but lacking memory. Lymphocytes are the key players of the adaptive immune system. Each lymphocyte expresses antigen-receptors of unique specificity. Upon recognizing an antigen via the receptor, lymphocytes proliferate and develop effector function. Few lymphocytes exhibit specificity for a given antigen or pathogen, and massive proliferation is usually required before an effector response can be measured - hence, the slow kinetics of the adaptive immune system. Since a significant proportion of the expanded lymphocytes survive and may maintain some effector function following elimination of the antigen,

the adaptive immune system reacts faster when encountering the antigen a second time. This is the basis of its ability to remember.

[0003] In contrast to the situation with lymphocytes, where specificity for a pathogen is confined to few cells that must expand to gain function, the cells and molecules of the innate immune system are usually present in massive numbers and recognize a limited number of invariant features associated with pathogens (Medzhitov, R. and Janeway, C.A., Jr., *Cell* 91:295-298 (1997)). Examples of such patterns include lipopolysaccharides (LPS), non-methylated CG-rich DNA (CpG) or double stranded RNA, which are specific for bacterial and viral infections, respectively.

[0004] Most research in immunology has focused on the adaptive immune system and only recently has the innate immune system entered the focus of interest. Historically, the adaptive and innate immune system were treated and analyzed as two separate entities that had little in common. Such was the disparity that few researchers wondered why antigens were much more immunogenic for the specific immune system when applied with adjuvants that stimulated innate immunity (Sotomayor, E. M., *et al.*, *Nat. Med.* 5:780 (1999); Diehl, L., *et al.*, *Nat. Med.* 5:774 (1999); Weigle, W. O., *Adv. Immunol.* 30:159 (1980)). However, the answer posed by this question is critical to the understanding of the immune system and for comprehending the balance between protective immunity and autoimmunity.

[0005] Stimulation of innate immunity alone is able to confer non-specific protection from infection, mainly via induction of cytokines. In addition, topical and local application of stimulators of innate immunity may be able to protect from tumor growth. DNA rich in non-methylated CG motifs (CpG), as present in bacteria and most non-vertebrates, is an important example of such a stimulator of innate immunity, since CpGs exhibit a potent stimulatory activity on B cells, dendritic cells and other APC's *in vitro* as well as *in vivo*. Although bacterial DNA is immunostimulatory across many vertebrate species, the individual CpG motifs may differ. In fact, CpG motifs that

stimulate mouse immune cells may not necessarily stimulate human immune cells and vice versa.

[0006] Interestingly, two types of CpGs exist, those that activate B cells and trigger the production of IL-12 (B-type) and those that activate plasmacytoid DCs and induce the production of IFN $\alpha$ . In general, B-type CpGs exhibit maximal activity only if the natural phosphodiester bond of the DNA is replaced by non-natural phosphothioester bond. This modification not only stabilizes the CpGs and protects them from degradation by nucleases but also leads to enhanced recognition by TLR9. This is different for A-type CpGs, which are optimally recognized by TLR9 in their natural phosphodiester form, while phosphothioester stabilized A-type CpGs are poorly recognized (Krieg AM, Annu Rev Immunol. 2002;20:709-60).

[0007] Therefore, in usefulness of A-type CpGs is often limited *in vivo*, since they are rather unstable *in vivo*. Thus, they exhibit unfavourable pharmacokinetics. In order to render A-type CpG-oligonucleotides more potent, it would be essential to apply them in a protected form. One possibility to stabilize A-type CpGs is to package them into virus-like particles (VLPs), which protect them from degradation (WO03/024481). However, this leads to a concomitant strong T and B cell response against the VLPs. While this is desirable if the VLPs are used as vaccines, this is a disadvantage for non-specific stimulation of innate immunity, since it precluded multiple applications.

[0008] It has previously been shown that application of B-type CpGs in liposomes enhances their capacity to induce production of IL-12 *in vitro* and *in vivo* (J Immunol 167: 3324). However, liposomes were reported not to enhance the potency of A-type CpGs (WO 03/040308 2A). We now found surprisingly, that liposomes strongly enhance the *in vivo* efficacy of a particular type of CpG, G10 (SEQ ID NO: 3), and shorter versions of it.

## SUMMARY OF THE INVENTION

[0009] This invention is based on the surprising finding that liposomes not only enhance the *in vivo* efficacy of B-type CpGs but also of the G10 CpG, the prototype A-type CpG. This now offers the unexpected opportunity to induce high levels of IFN $\alpha$  *in vivo* using A-type CpGs.

[0010] In a first embodiment, the invention provides a composition for inducing the production of IFN $\alpha$  in an animal comprising a liposome and an unmethylated CpG-containing oligonucleotide, where the oligonucleotide is bound to or enclosed by the liposome.

[0011] In a preferred embodiment of the invention, the immunostimulatory nucleic acids, is an A-type (also called D-type) CpG. In a most preferred embodiment, the CpG has the sequence gggggggggggacgatcgtcgaaaaaaa (SEQ ID NO: 3) or is a shorter version thereof.

[0012] In a preferred embodiment, the liposome is neutral, anionic, cationic, stealth or cationic stealth. In a most preferred embodiment, the liposome is a cationic liposome. In a further preferred embodiment the liposome is smaller than 200 nm.

[0013] The route of injection is preferably subcutaneous or intramuscular, but it would also be possible to apply the A-type CpG-containing liposomes intradermally, intranasally, intravenously or directly into the lymph node. In an equally preferred embodiment, the A-type CpG-containing liposomes mixed with antigen are applied locally, near a tumor or local viral reservoir.

[0014] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

#### BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0015] Figure 1 shows that phosphodiester (type A) oligonucleotides efficiently activate human CD8+ T cells from peripheral blood. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood of healthy volunteers by ficoll (Amersham Biosciences, Sweden) density centrifugation. PBMC were resuspended in 10% FCS RPMI and plated in 96-U-bottom well plate at  $0.3 \times 10^6$  cells/well. Cells were treated with the indicated concentrations of oligonucleotides or left untreated for 24h, at 37°C. PBMC were stained on ice with a combination of anti-CD8-FITC and anti-CD69-APC (all from Becton Dickinson, USA). Cells were acquired and analyzed using FACSCalibur (Becton Dickinson, USA).

[0016] Figure 2 shows that phosphothioate (type B) oligonucleotides efficiently activate human B cells. Peripheral blood mononuclear cells were (PBMC) obtained from heparinized blood of healthy volunteers by ficoll (Amersham Biosciences, Sweden) density centrifugation. PBMC were resuspended in 10% FCS RPMI and plated in 96-U-bottom well plate at  $0.3 \times 10^6$  cells/well. Cells were treated with the indicated concentrations of oligonucleotides or left untreated for 24h, at 37°C. PBMC were stained on ice with a combination of anti-CD19-PE and anti-CD69-APC (all from Becton Dickinson, USA). Cells were acquired and analyzed using FACSCalibur (Becton Dickinson, USA).

[0017] Figure 3 shows that only phosphodiester (Type A) oligonucleotides induce IFN alpha secretion from human PBMC. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood of healthy volunteers by ficoll (Amersham Biosciences, Sweden) density centrifugation. PBMC were resuspended in 10% FCS RPMI and plated in 96-U-bottom well plate at  $0.3 \times 10^6$  cells/well. Cells were treated with the indicated concentrations of oligonucleotides or left untreated for 24h, at 37°C. IFN alpha, released in the supernatants was measured by ELISA using an antibody set (Cat. # 71100-1) from PBL Biomedical Laboratories, USA.

[0018] Figure 4 shows that phosphothioester (type B) oligonucleotides induce IL-12 secretion from human PBMC. Peripheral blood mononuclear cells were (PBMC) obtained from heparinized blood of healthy volunteers by ficoll (Amersham Biosciences, Sweden) density centrifugation. PBMC were resuspended in 10% FCS RPMI and plated in 96-U-bottom well plate at  $0.3 \times 10^6$  cells/well. Cells were treated with the indicated concentrations of oligonucleotides or left untreated for 24h, at 37°C. IL-12, released in the supernatants was measured by ELISA using an antibody pair provided from Becton Dickinson (C8.3 and C8.6 clones).

[0019] Figure 5 shows that phosphodiester (type A) oligonucleotides induce IFN alpha secretion from human plasmacytoid DC (pDC). pDC were isolated from human PBMC by magnetic activated cell sorting (MACS). PBMC from buffy coats were labeled with anti-BDCA-2 mAb coupled to magnetic beads (Milteniy, Germany) according to manufacturer's protocol. Labeled cells were positively selected by passing PBMC through a LS column. The purity of pDC was controlled by staining them with anti-BDCA-4-APC mAb (Milteniy). pDC were plated at  $0.04 \times 10^6$ /well and treated with G10, 2006 or left untreated. Twenty four hours later IFN alpha released in the supernatants was measured by ELISA, as described in the legend of figure 3.

[0020] Figure 6 shows that phosphothioester -stabilized G10 (G10 PS) fails to activate human T cells. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood of healthy volunteers by ficoll (Amersham Biosciences, Sweden) density centrifugation. PBMC were resuspended in 10% FCS RPMI and plated in 96-U-bottom well plate at  $0.3 \times 10^6$  cells/well. Cells were treated with the indicated concentrations of oligonucleotides or left untreated for 24h, at 37°C. IFN alpha, released in the supernatants was measured by ELISA using an antibody set (Cat. # 71100-1) from PBL Biomedical Laboratories, USA.

[0021] Figure 7 shows that 1668pt but not 1668po or G6 is able to enhance CTL responses in vivo. Fig. 7(A): Mice were immunized with 100 ug of p33-VLPs (HBcAg with genetically fused the p33 epitope) alone or mixed with

1668pt or 1668po CpGs (20 nmol). Twelve days later, mice were challenged ip with recombinant vaccinia virus expressing LCMV GP ( $1 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later. Fig. 7(B): The bacteriophage Q $\beta$  capsid was used as VLP, to which the p33 peptide was chemically coupled, and co-delivered with the G6 CpG. Mice were left untreated or immunized with 90 ug of Q $\beta$  p33-VLPs mixed with G6 CpGs (20 nmol). Twelve days later, mice were challenged ip with recombinant vaccinia virus expressing LCMV GP ( $1 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later.

[0022] Figure 8 shows that G6 in liposomes is able to enhance p33-specific immunity. Fig. 8(A): Liposomes containing 1 mg/ml p33 peptide (KAVYNFATM) alone or with 100 nmol/ml CpGs (ODN1668 or ODNG6) were produced. Subsequently, groups of C57BL/6 mice were vaccinated with the liposomal preparations (doses of 100 ug p33 peptide alone or with 10 nmol ODN1668 or ODNG6 per mouse) and p33-specific T cell responses were assessed by tetramer-staining 8 days later. Fig. 8(B): At day 12, liposome-treated mice were challenged ip with recombinant vaccinia virus expressing LCMV-GP ( $4 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later.

#### DETAILED DESCRIPTION OF THE INVENTION

[0023] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are hereinafter described.

1. Definitions

[0024] Animal: As used herein, the term "animal" is meant to include, for example, humans, sheep, horses, cattle, pigs, dogs, cats, rats, mice, birds, reptiles, fish, insects and arachnids.

[0025] Antibody: As used herein, the term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant. The term is meant to include whole antibodies and antigen-binding fragments thereof, including single-chain antibodies. Most preferably the antibodies are human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V<sub>L</sub> or V<sub>H</sub> domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit, goat, guinea pig, camel, horse or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

[0026] The compositions and methods of the invention are also useful for treating cancer by stimulating non-specific immunity against cancer which may enhance specific immunity against tumor antigens. A "tumor antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer and which is capable of provoking an immune response. In particular, the compound is capable of provoking an immune response when presented in the context of an MHC molecule. Tumor antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, *et al.*, *Cancer Research*, 54:1055 (1994), by partially purifying the antigens, by recombinant technology or by de novo synthesis of known antigens. Tumor antigens include antigens that are antigenic portions of or are a whole tumor or cancer polypeptide. Such antigens can be isolated

or prepared recombinantly or by any other means known in the art. Cancers or tumors include, but are not limited to, biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas.

[0027] Antigenic determinant: As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes responding to antigenic determinants produce antibodies, whereas T-lymphocytes respond to antigenic determinants by proliferation and establishment of effector functions critical for the mediation of cellular and/or humoral immunity.

[0028] Antigen presenting cell: As used herein, the term "antigen presenting cell" is meant to refer to a heterogeneous population of leucocytes or bone marrow derived cells which possess an immunostimulatory capacity. For example, these cells are capable of generating peptides bound to MHC molecules that can be recognized by T cells. The term is synonymous with the term "accessory cell" and includes, for example, Langerhans' cells, interdigitating cells, dendritic cells, B cells and macrophages. Under some conditions, epithelial cells, endothelial cells and other, non-bone marrow derived cells may also serve as antigen presenting cells.

[0029] Bound: As used herein, the term "bound" refers to binding that may be covalent, e.g., by chemically coupling the unmethylated CpG-containing oligonucleotide to a liposome, or non-covalent, e.g., ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term also includes the enclosure, or partial enclosure, of a substance. The term "bound" is broader than and includes terms such as "coupled," "fused," "enclosed" and

"attached." Moreover, with respect to the immunostimulatory substance being bound to the liposome, the term "bound" also includes the enclosure, or partial enclosure, of the immunostimulatory substance. Therefore, with respect to the immunostimulatory substance being bound to the liposome the term "bound" is broader than and includes terms such as "coupled," "fused," "enclosed", "packaged" and "attached." For example, the immunostimulatory substance such as the unmethylated CpG-containing oligonucleotide can be enclosed by the liposome without the existence of an actual binding, neither covalently nor non-covalently, such that the oligonucleotide is held in place by mere "packaging."

[0030] CpG: As used herein, the term "CpG" refers to an oligonucleotide which contains at least one unmethylated cytosine, guanine dinucleotide sequence (*e.g.* "CpG-oligonucleotides" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates/activates, *e.g.* has a mitogenic effect on, or induces or increases cytokine expression by, a vertebrate bone marrow derived cell. For example, CpGs can be useful in activating B cells, NK cells and antigen-presenting cells, such as dendritic cells, monocytes and macrophages. The CpGs can include nucleotide analogs such as analogs containing phosphorothioester bonds and can be double-stranded or single-stranded. Generally, phosphorothioester stabilized CpGs are B-type CpGs while phosphodiester CpGs are A-type CpGs.

[0031] A-type CpGs: As used herein, the term "A-type CpG" refers to CpGs which preferentially stimulate activation of T cells if human blood cells are stimulated and induce the release of IFN $\alpha$ .

[0032] B-type CpGs: As used herein, the term "B-type CpG" refers to CpGs which preferentially stimulate activation of B cells if human blood cells are stimulated and induce the release of IL-12. The distinction between A-type and B-type CpGs may not be absolute but one type of response (*i.e.* release of IL-12 versus IFN $\alpha$ ) usually dominates.

[0033] G10: As used herein, the term "G10" refers to the sequence GGGGGGGGGGGACGATCGTCGGGGGGGGGG (SEQ ID NO: 3). However, throughout the text, G10 also includes shorter versions of the molecule or longer oligomers that contain the G10 motif or shorter versions thereof. CpG sequences used in the present invention are shown in table 1.

[0034] Immune response: As used herein, the term "immune response" refers to the systemic or local production of cytokines/chemokines/interferons

[0035] Immunization: As used herein, the terms "immunize" or "immunization" or related terms refer to conferring the ability to mount a substantial immune response (including non-specific production of cytokines, chemokines, interferons and alike). These terms do not require that complete immunity be created, but rather that an immune response be produced which is substantially greater than baseline. For example, a mammal may be considered to be immunized if systemic or local cytokine/chemokine/interferon production can be measured.

[0100] Immunostimulatory nucleic acid: As used herein, the term immunostimulatory nucleic acid refers to a nucleic acid capable of inducing and/or enhancing an immune response. Immunostimulatory nucleic acids, as used herein, comprise ribonucleic acids and in particular deoxyribonucleic acids. Preferably, immunostimulatory nucleic acids contain at least one CpG motif e.g. a CG dinucleotide in which the C is unmethylated. The CG dinucleotide can be part of a palindromic sequence or can be encompassed within a non-palindromic sequence. Immunostimulatory nucleic acids not containing CpG motifs as described above encompass, by way of example, nucleic acids lacking CpG dinucleotides, as well as nucleic acids containing CG motifs with a methylated CG dinucleotide. The term "immunostimulatory nucleic acid" as used herein should also refer to nucleic acids that contain modified bases such as 4-bromo-cytosine.

[0036] Pathogen: Pathogens include, but are not limited to, infectious virus, infectious bacteria, parasites and infectious fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives

thereof and also synthetic or recombinant compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to the skilled artisan.

[0037] Examples of infectious viruses that have been found in humans include but are not limited to: Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III); and other isolates, such as HIV-LP); Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronoviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

[0038] Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, Pasteurella species, Staphylococci species and Streptococcus species. Gram negative bacteria include, but are not limited to, Escherichia coli, Pseudomonas species, and Salmonella species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophila*, Mycobacteria sps. (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus antracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, *Actinomyces israelli* and *Chlamydia*.

[0039] Examples of infectious fungi include: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis* and *Candida albicans*. Other infectious organisms (i.e., protists) include: Plasmodium such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, *Toxoplasma gondii* and *Shistosoma*.

[0040] Other medically relevant microorganisms have been described extensively in the literature, e.g., see C. G. A. Thomas, "Medical Microbiology", Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

[0041] Effective Amount: As used herein, the term "effective amount" refers to an amount necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves this selected result, and such an amount could be determined as a matter of routine by a person skilled in the art. For example, an effective amount for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in the production of cytokines and alike. The term is also synonymous with "sufficient amount."

[0042] The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the size of the subject, and/or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular composition of the present invention without necessitating undue experimentation.

[0043] Treatment: As used herein, the terms "treatment", "treat", "treated" or "treating" refer to prophylaxis and/or therapy. When used with respect to an infectious disease, for example, the term refers to a prophylactic treatment which increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen or will show signs of illness attributable to the infection, as well as a treatment after the subject has become infected in order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse.

[0044] One, a, or an: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

[0045] As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning, polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies are well known to those skilled in the art and can be

conveniently found in published laboratory methods manuals (e.g., Sambrook, J. et al., eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. et al., eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)). Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth. Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., "Protein Purification Principles and Practice," 3<sup>rd</sup> ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

2. Compositions and Methods for Enhancing of CpG-induced IFN $\alpha$ -production by liposomes:

[0046] The disclosed invention provides compositions and methods for enhancing the production of IFN $\alpha$  by CpGs in an animal. Compositions of the invention comprise, or alternatively consist of, a liposome and an unmethylated CpG-containing oligonucleotide where, preferably of the A-type and more preferably the CpG is G10 and the oligonucleotides are bound to or enclosed by the liposome. Furthermore, the invention conveniently enables the practitioner to construct such a composition for various treatment and/or prevention purposes, which include the prevention and/or treatment of infectious diseases, as well as chronic infectious diseases, the prevention and/or treatment of cancers.

[0047] In a further preferred embodiment, the oligonucleotide may be a shorter version of G10, such as GGGGACGATCGTCGGGGG (SEQ ID NO: 6); GGGGGACGATCGTCGGGGG (SEQ ID NO: 7); GGGGGGACGATCGTCGGGGG (SEQ ID NO: 8); GGGGGGGACGATCGTCGGGGG (SEQ ID NO: 9);

GGGGGGGGGACGATCGTCGGGGGGG (SEQ ID NO:10);  
GGGGGGGGGACGATCGTCGGGGGGG (SEQ ID NO: 11);  
GGGGGGGGGACGATCGTCGGGGGGG (SEQ ID NO: 12); and  
GGGGGGCGACGACGATCGTCGTGGGGGG (SEQ ID NO: 5).

[0048] In a further preferred embodiment, the oligonucleotides may contain the G10 sequence or a shorter version thereof.

[0049] Liposomes in the context of the present application refer to lipid vesicles consisting of a lipid bilayer that can be used to entrap or bind various drugs including CpGs.

[0050] In a preferred embodiment, the liposome exhibits positive charges in order to facilitate interaction of T cells with target cells. Generation of such liposomes is well established, for details see eg J Immunol 167: 3324 and references therein.

[0101] The G10 oligonucleotide can also be recombinant, genomic, synthetic, cDNA, plasmid-derived and single or double stranded. For use in the instant invention, the nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the *b*-cyanoethyl phosphoramidite method (Beaucage, S. L., and Caruthers, M. H., *Tet. Let.* 22:1859 (1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054 (1986); Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407 (1986); Garegg *et al.*, *Tet. Let.* 27:4055-4058 (1986), Gaffney *et al.*, *Tet. Let.* 29:2619-2622 (1988)). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, CpGs can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides. Oligonucleotides can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

[0102] In one aspect of the invention, the A-type CpGs in liposomes are used to induce systemically increased levels of IFN $\alpha$ . Such elevated levels of IFN $\alpha$

are known to be therapeutically active during hepatitis B and hepatitis C virus infection and perhaps also during infection with HIV. Moreover, IFNa non-specifically protects from viral and some bacterial infection, rendering A-type CpGs in liposomes ideal prophylactic "non-specific" vaccines against infections in general. In addition, local application of A-type CpGs, as eg injection into tumors, has been shown to protect from tumor growth. Thus, A-type CpGs in liposomes may be particularly attractive for the treatment of cancer.

[0103] The invention also provides vaccine compositions which can be used for preventing and/or attenuating diseases or conditions. Vaccine compositions of the invention comprise, or alternatively consist of, an immunologically effective amount of the inventive immune enhancing composition together with a pharmaceutically acceptable diluent, carrier or excipient. The vaccine can also optionally comprise an adjuvant.

[0104] The invention further provides vaccination methods for preventing and/or attenuating diseases or conditions in animals. In one embodiment, the invention provides vaccines for the prevention of infectious diseases in a wide range of animal species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines can be designed to treat infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc.

[0105] In another embodiment, the invention provides vaccines for the prevention of cancer in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines can be designed to treat all types of cancer including, but not limited to, lymphomas, carcinomas, sarcomas and melanomas.

[0106] As would be understood by one of ordinary skill in the art, when compositions of the invention are administered to an animal, they can be in a

composition which contains salts, buffers, adjuvants or other substances which are desirable for improving the efficacy of the composition. Examples of materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including REMINGTON'S PHARMACEUTICAL SCIENCES (Osol, A, ed., Mack Publishing Co., (1990)).

[0107] The compositions of the present invention can be administered by various methods known in the art. The particular mode selected will depend of course, upon the particular composition selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, can be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, parenteral, intracisternal, intravaginal, intraperitoneal, topical (as by powders, ointments, drops or transdermal patch), bucal, or as an oral or nasal spray. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. The composition of the invention can also be injected directly in a lymph node.

[0108] Dosage levels depend on the mode of administration, the nature of the subject, and the quality of the carrier/adjuvant formulation. Typical amounts are in the range of about 0.1 µg to about 100 mg CpG per subject. Preferred amounts are at least about 10 µg to about 1000 µg per subject. Multiple administration to immunize the subject is preferred, and protocols are those standard in the art adapted to the subject in question.

[0109] The compositions can conveniently be presented in unit dosage form and can be prepared by any of the methods well-known in the art of pharmacy. Methods include the step of bringing the compositions of the invention into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compositions of the invention into association with a liquid

carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

- [0110] Compositions suitable for oral administration can be presented as discrete units, such as capsules, tablets or lozenges, each containing a predetermined amount of the compositions of the invention. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, an elixir or an emulsion.
- [0111] Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compositions of the invention described above, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art.
- [0112] Other embodiments of the invention include processes for the production of the compositions of the invention and methods of medical treatment for cancer and allergies using said compositions.
- [0113] The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.
- [0114] All patents, patent applications and publications referred to herein are expressly incorporated by reference in their entirety.

#### EXAMPLES

Table 1: Terminology and sequences of immunostimulatory nucleic acids used in the Examples.

Small letters indicate deoxynucleotides connected via phosphorothioate bonds while large letters indicate deoxynucleotides connected via phosphodiester bonds

Terminology	Sequence	SEQ ID NO
CpG 1668	tccatgacgttcctgaataat	1
CpG-2006	tcgtcgtttgcgtttgtcgt	2
G10	GGGGGGGGGGGACGATCGTCGGGGGGGGGG	3
G10-PS	gggggggggggacgtcggggggggggg	4
G6	GGGGGGCGACGACGATCGTCGTGGGGGGGG	5
G3-6	GGGGACGATCGTCGGGGGGGG	6
G4-6	GGGGGACGATCGTCGGGGGGGG	7
G5-6	GGGGGGACGATCGTCGGGGGGGG	8
G6-6	GGGGGGGACGATCGTCGGGGGGGG	9
G7-7	GGGGGGGGACGATCGTCGGGGGGGGGG	10
G8-8	GGGGGGGGGACGATCGTCGGGGGGGGGG	11
G9-9	GGGGGGGGGGACGATCGTCGGGGGGGGGG	12

#### EXAMPLE 1

G10 and analogues activate T cells in human blood cultures more efficiently than CpG 2006

[0115] Human peripheral blood mononuclear cells (PBMC) were isolated and stimulated with various concentrations of CpG G10, G9-9, G8-8, G7-7 or the thioester stabilized CpG 2006. The next day, cells were stained for the expression of CD8 and CD69 in order to test for T cell activation. G10, G9-9, G8-8, G7-7 all efficiently activated CD8+ T cells, with G10 and G9-9 being most effective while G7-7 was least effective. In contrast, 2006 was barely able to activate human T cells (Fig 1). This characterizes G10, G9-9, G8-8, G7-7 as A type CpGs while 2006 is characterized as a B type CpG.

#### EXAMPLE 2

2006 but not G10 and analogues activate B cells in human blood cultures

[0116] Human PBMC were isolated and stimulated with various concentrations of CpG G10, G9-9, G8-8, G7-7 or the thioester stabilized CpG 2006. The next day, cells were stained for the expression of CD19 and CD69 in order to test for B cell activation. G10, G9-9, G8-8, G7-7 failed to efficiently activate B cells. In contrast, 2006 was very effective at activating human B cells. This characterizes G10, G9-9, G8-8, G7-7 as A type CpGs while 2006 is characterized as a B type CpG.

#### EXAMPLE 3

G10 and analogues but not CpG 2006 induce production of IFN $\alpha$  in human PBMC

[0117] Human PBMC were isolated and stimulated with various concentrations of CpG G10, G9-9, G8-8, G7-7, G3, G6, G4-6 and G6-6 or the thioester stabilized CpG 2006. 24h later, supernatants were assessed for the presence of IFN $\alpha$  by ELISA. G10, G9-9, G8-8, G7-7, G3, G6, G4-6 and G6-6 all efficiently induced the production of IFN $\alpha$ , with G10 being most effective while G4-6 least effective. In contrast, 2006 was not able to induce IFN alpha release from human PBMC (Fig 3). This characterizes G10, G9-9, G8-8, G7-7 as A type CpGs while 2006 is characterized as a B type CpG.

#### EXAMPLE 4

2006 and 1668 but not G10 induce production of IL-12 in human blood cultures

[0118] Human blood cells were isolated stimulated with various concentrations of CpG G10 or the thioester stabilized CpG 2006 or 1668. 24h later, presence of IL-12 was assessed in the supernatant by ELISA. G10 failed to induce production of IL-12 while both thioesterstabilzed CpGs efficiently triggered the release of IL-12 (Fig 4). This characterizes G10 as A type CpGs while 2006 and 1668 are characterized as a B type CpG.

#### EXAMPLE 5

G10 but not 2006 induces production of IFN $\alpha$  in human plasmacytoid DCs

[0119] Human plasmacytoid DCs (pDCs) were isolated from PBMC by labeling them with anti-BDCA-2 mAb attached to magnetic beads (Miltenyi Biotec, Germany). pDCs were subsequently stimulated with the CpGs G10 or the phosphothioester stabilized CpG 2006 (20 nM) and release of IFN $\alpha$  into the supernatant was monitored subsequently by ELISA. Only G10 but not 2006 was able to efficiently trigger release of IFN $\alpha$  (Fig 5).

#### EXAMPLE 6

Phosphothioester stabilized G10 (G10-PS) fails to stimulate T cells in human blood cultures

[0120] Human blood cells were isolated and stimulated with various concentrations of CpG G10 or the thioester stabilized CpG G10 (G10-PS). 24h later IFN alpha released in the supernatants was measured by ELISA. G10 efficiently induced production of IFN alpha, while the thioester stabilized version was barely active. 2006 failed to induce IFN alpha secretion (Fig 6). Thus, thioester-stabilized G10 (G10-PS) does not behave as an A-type CpG.

#### EXAMPLE 7

1668pt but not 1668po or G6 is able to enhance CTL responses in vivo

[0121] CpGs are able to non-specifically activate antigen-presenting cells. However, *in vivo*, usually only thioester-stabilized oligonucleotides may be active. We have previously observed that thioester stabilized CpGs are able to enhance CTL responses *in vivo* if mixed together with VLPs (J Immunol. 168: 2880). We now compared the ability of 1668pt (B type) CpGs with 1668po (A-type) CpGs to enhance CTL responses upon mixing with VLPs. As a model VLP, hepatitis B core Ag fused to peptide p33 derived from LCMV was used. The p33-VLPs were generated as follows: Hepatitis B clone pEco63 containing the complete viral genome of Hepatitis B virus was purchased from ATCC. The gene encoding HBcAg was introduced into the EcoRI/HindIII restriction sites of expression vector pKK223.3 (Amersham Pharmacia Biotech Inc., NJ) under the control of a tac promotor. The p33 peptide (KAVYNFATM, SEQ ID NO: 13) derived from LCMV was fused to the C-terminus of HBcAg (aa 1-183) via a three leucine-linker by standard PCR methods. *E. coli* K802d were transfected with the plasmid and grown in 2 liter cultures until an optical density of 1 (at 600 nm wavelength). Cells were induced by adding IPTG (Sigma, Division of Fluka AG, Switzerland) to a final concentration of 1mM for 4 hours. Bacteria were then collected by centrifugation and resuspended in 5 ml lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 30 mM NaCl, 10 mM EDTA, 0.25 % Tween-20, pH 7.0). 200 µl of lysozyme solution (20 mg/ml) was added. After sonication 4 µl benzonase (Merck, Darmstadt, Germany) and 10 mM MgCl<sub>2</sub> were supplemented to the cell lysate. The suspension was then incubated for 30 minutes at RT and centrifuged for 15 minutes at 27000 x g. The retained supernatant was complemented with 20 % (w/v) ammonium sulfate. After incubation for 30 minutes on ice and centrifugation for 15 minutes at 48000 x g the supernatant was discarded and the pellet resuspended in 2-3 ml phosphate-saline buffer. The preparation was loaded onto a Sephadryl S-400 gel filtration column (Amersham Pharmacia

Biotech Inc., NJ) for purification. Fractions were analyzed for protein content in a SDS PAGE gel and samples containing pure HBc capsids were pooled.

[0122] Electron microscopy was performed according to standard protocols.

[0123] Mice were immunized with 100 µg of p33-VLPs alone or mixed with 1668pt or 1668po CpGs (20 nmol). Twelve days later, mice were challenged ip with recombinant vaccinia virus expressing LCMV GP ( $1 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later (J Immunol. 168: 2880) (Fig 7 A). Only 1668pt but not 1668po was able to enhance protective p33-specific CTL responses.

[0124] Alternatively, the bacteriophage Q $\beta$  capsid was used as VLP and co-delivered with the G6 CpG (Fig 7 B). Production and purification of Q $\beta$  is performed with the same protocol as for HBcAg VLPs. The p33 peptide was chemically coupled to the Q $\beta$  VLP via a bifunctional linker as follows: purified Q $\beta$  VLPs (1.5 mg/ml in 20 mM HEPES, 150 mM NaCl pH 7.2) were derivatized by a 30 min incubation at RT with a 10-fold molar excess of succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate (Pierce Biotechnology, Rockford, IL, USA). Free cross-linker was removed by extensive dialysis against 20 mM HEPES pH 7.2. Peptide p33 was produced in a modified version with three additional amino acids (GGC) added to the C-terminus (p33-GGC) (EMC microcollections GmbH, Tübingen, Germany) to allow coupling to VLPs. Derivatized Q $\beta$  VLPs and p33-GGC (peptide at 5-fold molar excess) were then incubated for 2 h at RT to allow cross-linking. Free p33-GGC was removed by dialysis against 20 mM HEPES pH 7.2 using DispoDialyser membranes with a molecular weight cut-off of 300 kD (Spectrum Medical Industries Inc., Rancho Dominguez, CA). Efficiency of cross-linking was analysed by SDS polyacrylamide gel electrophoresis.

[0125] Mice were left untreated or immunized with 90 µg of p33-VLPs mixed with G6 CpGs (20 nmol). Twelve days later, mice were challenged ip with recombinant vaccinia virus expressing LCMV GP ( $1 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later (J Immunol. 168: 2880) (Fig 7 B). G6 was not able to significantly induce protective p33-specific CTL responses.

#### EXAMPLE 8

##### G6 in liposomes is able to enhance p33-specific immunity

[0126] In order to test whether incorporation into liposomes may enhance the efficiency of G6, liposomes containing p33 and either G6 or 1668 were generated. Liposomes were produced as previously described (*Vaccine* 19, 23-32 (2000)). Briefly, small unilamellar liposomes were generated by freeze-thawing followed by sequential filter extrusion. The liposomal composition was 200 mg/ml soy phosphatidylcholine, 25 mg/ml cholesterol and 1.2 mg/ml DL- $\alpha$ -tocopherol. The dried lipid mixture was solubilized with 1 mg/ml p33 peptide (KAVYNFATM, SEQ ID NO: 13) alone or with 100 nmol/ml CpGs (ODN1668), subjected to 3-5 freeze-thaw cycles and repeatedly extruded through Nucleopore filters of 0.8, 0.4 and 0.2  $\mu$ m pore size (Sterico AG, Dietikon, Switzerland). Unencapsulated peptide and CpGs were removed by dialysis. Liposome size was determined by laser light scattering (Submicron Particle Sizer Model 370, Nicomp, Santa Barbara, USA). Mice were vaccinated subsequently with the liposomes and p33-specific T cell responses were assessed by tetramer-staining 8 days later (Fig 8A). At day 12, mice were challenged ip with recombinant vaccinia virus expressing LCMV-GP ( $4 \times 10^6$  pfu) and viral titers were determined in ovaries 5 days later (J Immunol. 168: 2880) (Fig 8B). Using liposomes, both 1668 and G6 were able to enhance protective p33-specific CTL responses.

#### EXAMPLE 9

##### G10 but not 2006 in liposomes is able to enhance production of IFN $\alpha$ in vivo

[0127] In order to test whether incorporation into liposomes may enhance the ability of G10 or 2006 to trigger the in vivo production of IFN $\alpha$ , liposomes containing p33 and either G10 or 1668 are generated. Liposomes are produced

as previously described (*Vaccine* 19, 23-32 (2000)). Briefly, small unilamellar liposomes are generated by freeze-thawing followed by sequential filter extrusion. The liposomal composition is 200 mg/ml soy phosphatidylcholine, 25 mg/ml cholesterol and 1.2 mg/ml DL- $\alpha$ -tocopherol. The dried lipid mixture is solubilized with 1 mg/ml or 50  $\mu$ g/ml p33 peptide (KAVYNFATM, SEQ ID NO: 13) alone or with 100 nmol/ml CpGs (ODN1668), subjected to 3-5 freeze-thaw cycles and repeatedly extruded through Nucleopore filters of 0.8, 0.4 and 0.2  $\mu$ m pore size (Sterico AG, Dietikon, Switzerland). Unencapsulated peptide and CpGs are removed by dialysis. Liposome size is determined by laser light scattering (Submicron Particle Sizer Model 370, Nicomp, Santa Barbara, USA). Mice are vaccinated subsequently with the liposomes and production of IFN $\alpha$  is analyzed 6, 12, 18 and 24 hours later in the blood of vaccinated mice.

**WHAT IS CLAIMED IS:**

1. A composition for enhancing an the production of IFN $\alpha$  in an animal comprising:
  - (a) Liposome;
  - (b) a A-type CpG;

wherein said A-type CpG (b) is bound to or entrapped by said liposome (a);
2. The composition of claim 1, wherein said A-type CpG is able to induce the secretion of IFN $\alpha$  in vitro.
3. The composition of claim 1, wherein said A-type CpG comprises the sequence as set forth in SEQ ID NO: 3.
4. The composition of claim 1, wherein said A-type CpG is derived from the sequence as set forth in SEQ ID NO: 3.
5. The composition of claim 1, wherein said A-type CpG has a nucleic acid sequence as set forth in SEQ ID NO: 3.
6. The composition of claim 1, wherein said A-type CpG has a nucleic acid sequence selected from
  - (a) GGGGACGATCGTCGGGGGG (SEQ ID NO: 6);
  - (b) GGGGGACGATCGTCGGGGGG (SEQ ID NO: 7);
  - (c) GGGGGGACGATCGTCGGGGGG (SEQ ID NO: 8);
  - (d) GGGGGGGACGATCGTCGGGGGG (SEQ ID NO: 9);
  - (e) GGGGGGGGACGATCGTCGGGGGGGG (SEQ ID NO:10);
  - (f) GGGGGGGGGACGATCGTCGGGGGGGG (SEQ ID NO: 11);
  - (g) GGGGGGGGGGACGATCGTCGGGGGGGG (SEQ ID NO: 12); and

(h) GGGGGGCGACGACGATCGTCGTGGGGGG (SEQ ID NO: 5).

7. The composition of claim 1, wherein said liposome is selected from the group of:

- (a) neutral,
- (b) anionic,
- (c) cationic,
- (d) stealth,
- (e) cationic stealth.

8. The composition of claim 1, wherein said liposome is a cationic liposome.

9. The composition of claim 1, wherein said A-type CpG comprises about 20 to about 300 nucleotides, preferably about 20 to about 100 nucleotides, and even more preferably about 20 to about 40 nucleotides.

10. The composition of claim 1, wherein said A-type CpG, is selected from

- (a) a recombinant oligonucleotide;
- (b) a genomic oligonucleotide;
- (c) a synthetic oligonucleotide;
- (d) a plasmid-derived oligonucleotide;
- (e) a PCR product;
- (f) a single-stranded oligonucleotide; and
- (g) a double-stranded oligonucleotide.

11. A method for enhancing the production of IFN $\alpha$  in an animal comprising introducing into said animal a composition comprising:
  - (a) liposome and
  - (b) A-type CpG bound to or entrapped by the liposome.
12. The method of claim 11, wherein said A-type CpG is able to induce the secretion of IFN $\alpha$  in vitro.
13. The method of claim 11, wherein said A-type CpG comprises the sequence as set forth in SEQ ID NO: 3.
14. The method of claim 11, wherein said A-type CpG is derived from the sequence as set forth in SEQ ID NO: 3.
15. The method of claim 11, wherein said A-type CpG has a nucleic acid sequence of as set forth in SEQ ID NO: 3.
16. The method of claim 11, wherein said A-type CpG has a nucleic acid sequence selected from
  - (a) GGGGACGATCGTCGGGGGG (SEQ ID NO: 6);
  - (b) GGGGGACGATCGTCGGGGGG (SEQ ID NO: 7);
  - (c) GGGGGGACGATCGTCGGGGGG (SEQ ID NO: 8);
  - (d) GGGGGGGACGATCGTCGGGGGG (SEQ ID NO: 9);
  - (e) GGGGGGGGACGATCGTCGGGGGGG (SEQ ID NO: 10);
  - (f) GGGGGGGGGACGATCGTCGGGGGGGG (SEQ ID NO: 11);
  - (g) GGGGGGGGGGACGATCGTCGGGGGGGG (SEQ ID NO: 12); and
  - (h) GGGGGGCGACGACGATCGTCGTGGGGGGG (SEQ ID NO: 5).

17. The method of claim 11, wherein said liposome is selected from the group of:
  - (a) neutral,
  - (b) anionic,
  - (c) cationic,
  - (d) stealth,
  - (e) cationic stealth.
18. The method of claim 11, wherein said liposome is a cationic liposome.
19. The method of claim 11, wherein said A-type CpG comprises about 20 to about 300 nucleotides, preferably about 20 to about 100 nucleotides, and even more preferably about 20 to about 40 nucleotides.
20. The method of claim 11, wherein said A-type CpG, is selected from
  - (a) a recombinant oligonucleotide;
  - (b) a genomic oligonucleotide;
  - (c) a synthetic oligonucleotide;
  - (d) a plasmid-derived oligonucleotide;
  - (e) a PCR product;
  - (f) a single-stranded oligonucleotide; and
  - (g) a double-stranded oligonucleotide.
21. The method of claim 11, wherein said animal is a mammal, preferably a human.
22. The method of claim 11, wherein said composition is introduced into said animal subcutaneously, intramuscularly, intravenously,

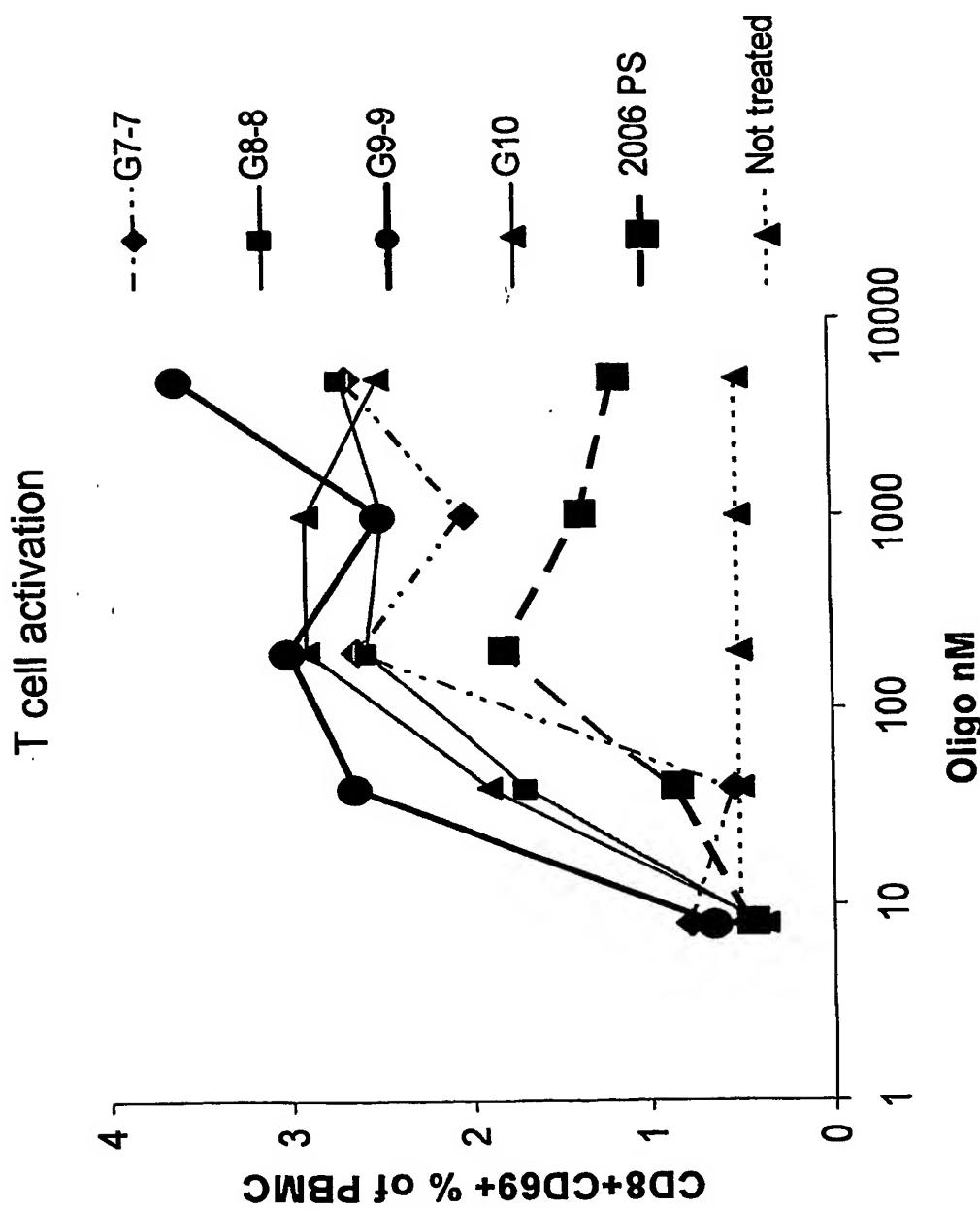
intranasally, directly into the lymph node or locally into onto or close to a tumor.

23. A vaccine comprising an immunologically effective amount of the composition of claim 1 together with a pharmaceutically acceptable diluent, carrier or excipient.
24. The vaccine of claim 23, further comprising an adjuvant.
25. A method of immunizing or treating an animal comprising administering to said animal an immunologically effective amount of the vaccine of claim 23.
26. The method of claim 21, wherein said animal is a mammal, preferably a human.
27. Use of a composition according to claim 1 or use of a vaccine according to claim 23 in the manufacture of a pharmaceutical for the treatment of a disorder or disease comprising, and preferably selected from the group consisting of cancer and infectious diseases.

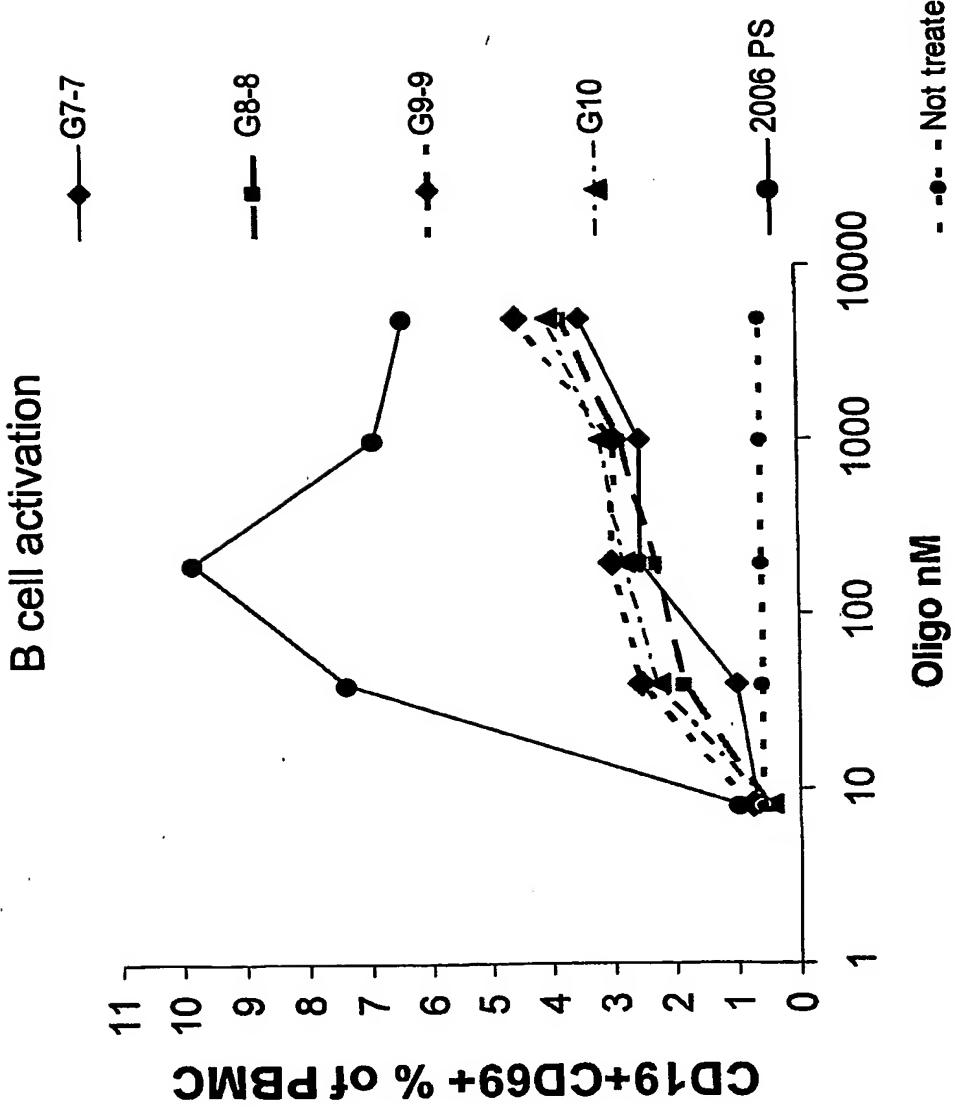
**Enhancement of A-Type CpG-Induced IFN $\alpha$ -Production by Liposomes:  
Method of Preparation and Use**

**ABSTRACT OF THE DISCLOSURE**

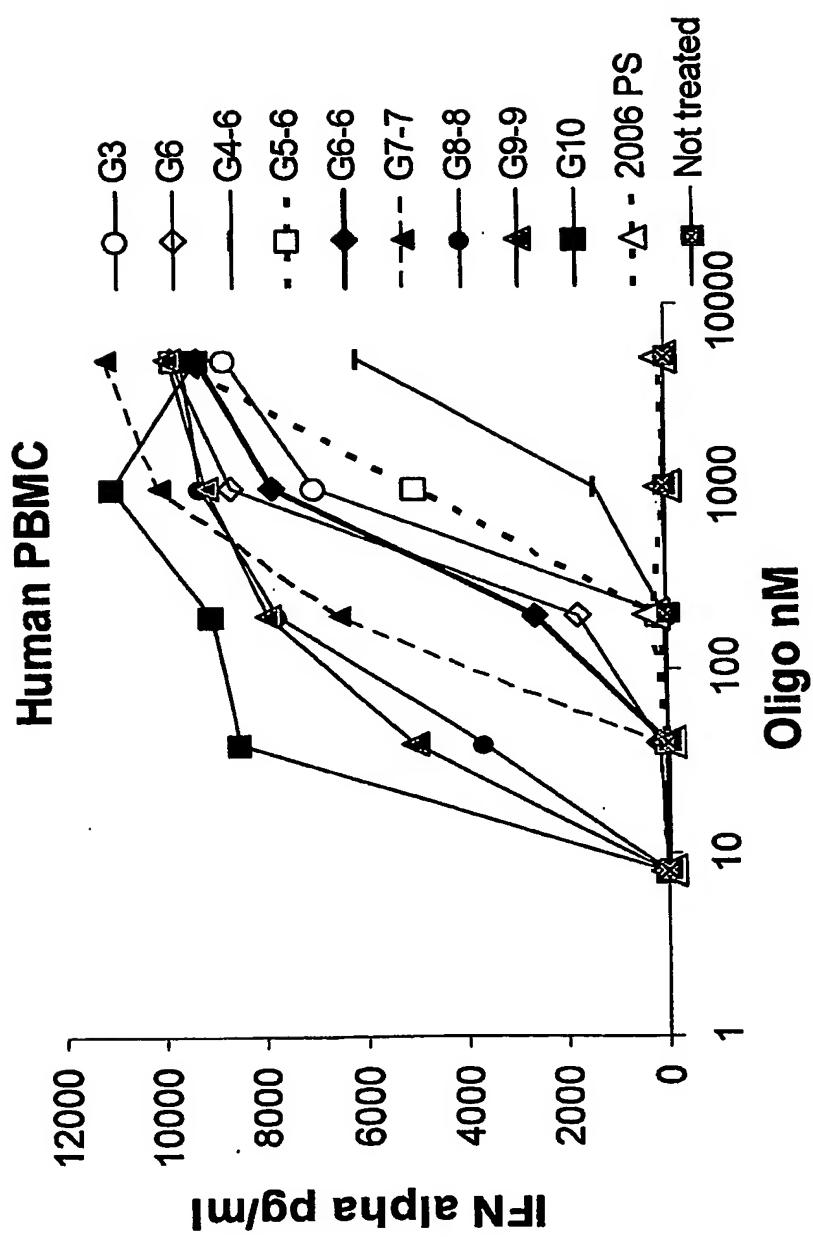
Liposomes are known to enhance the activity of K- (B-) type CpGs which trigger the production of IL-12. In the present invention, the surprising finding was made that liposomes also enhance the activity of D- (A-) type CpGs, leading to the production of IFN $\alpha$  in vivo. These findings are relevant for the humans situation, since IFN $\alpha$  rather than IL-12 is the key cytokine for the induction of Th1 responses and anti-viral protection in humans.



**Fig. 1**



**Fig. 2**



**Fig. 3**

IL-12 released by CpG-treated human PBMC

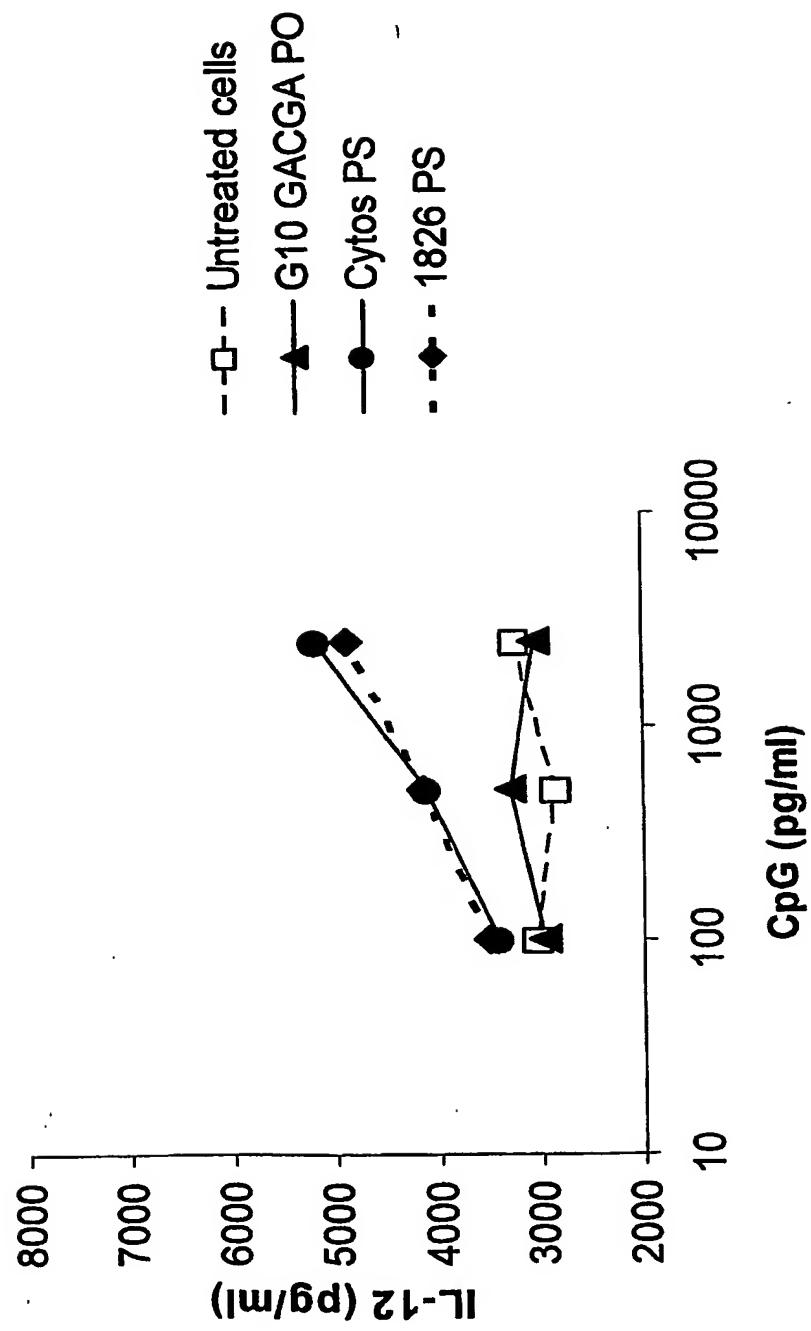
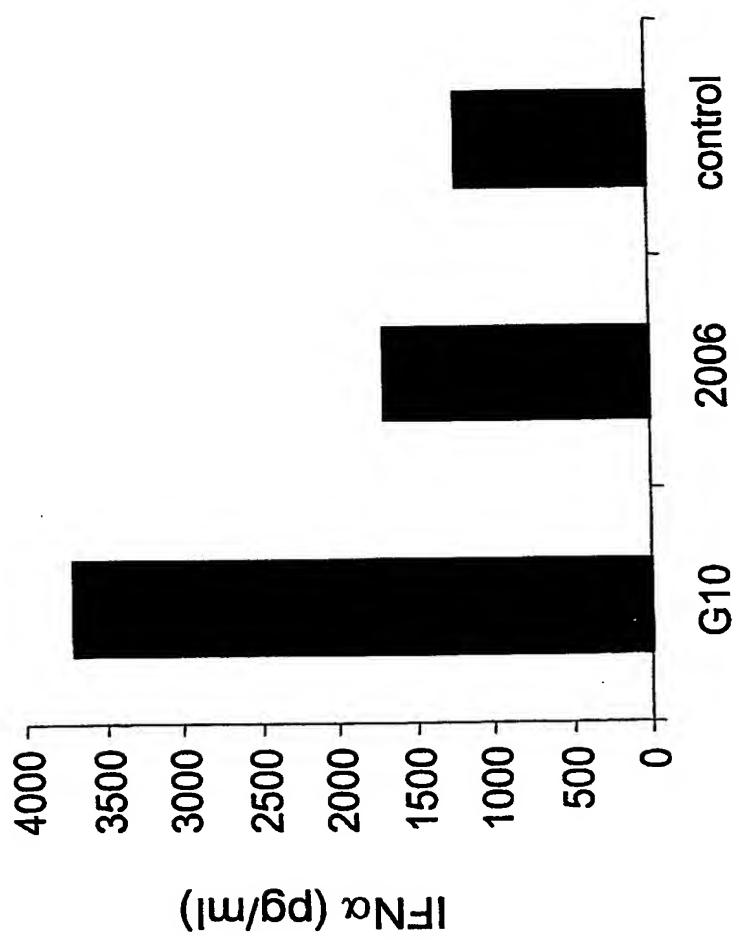
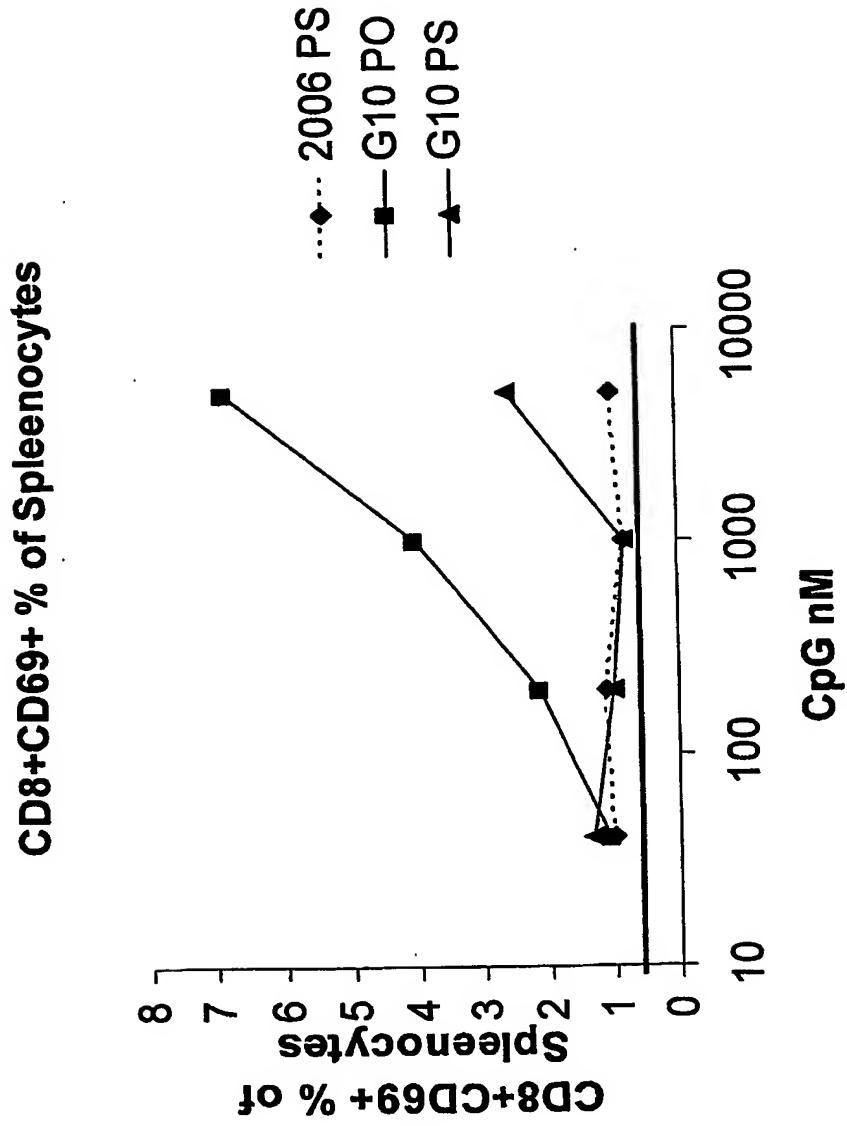


Fig. 4

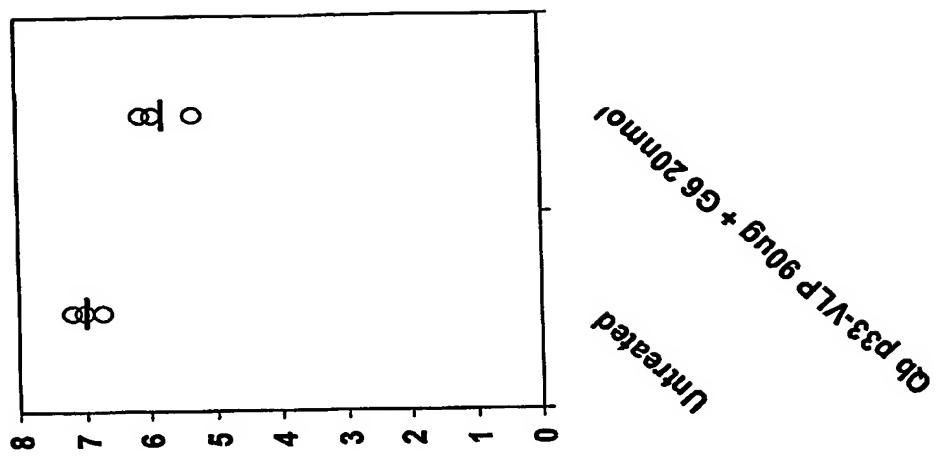


**Fig. 5**

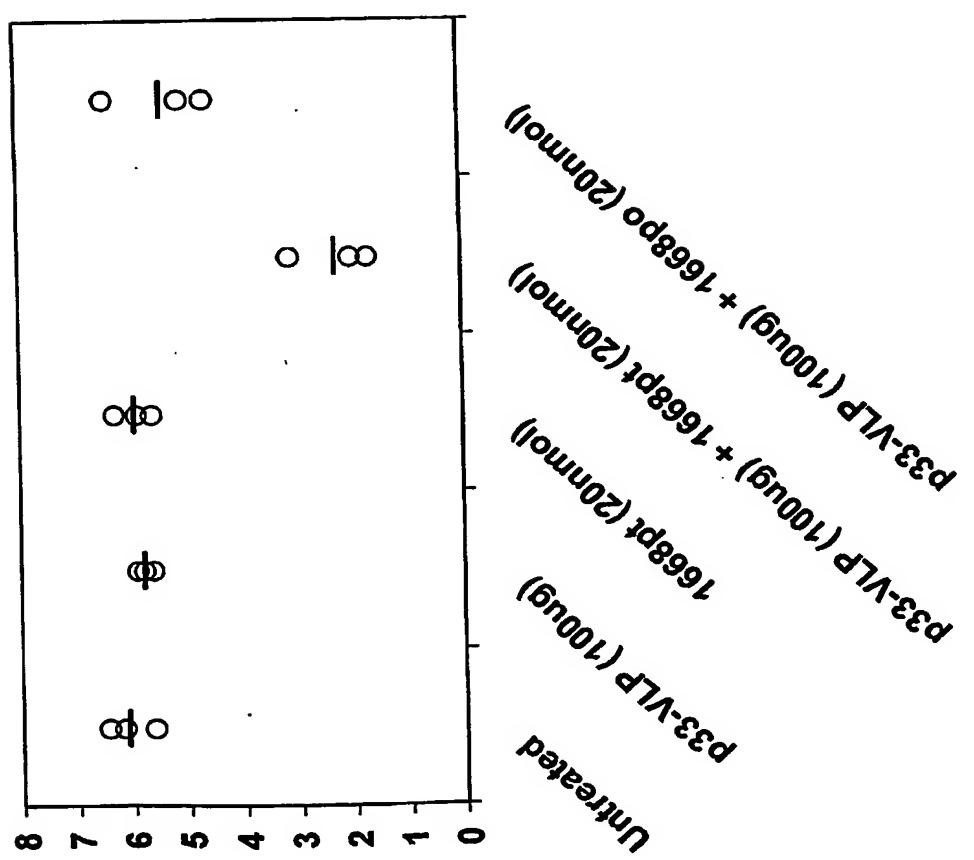


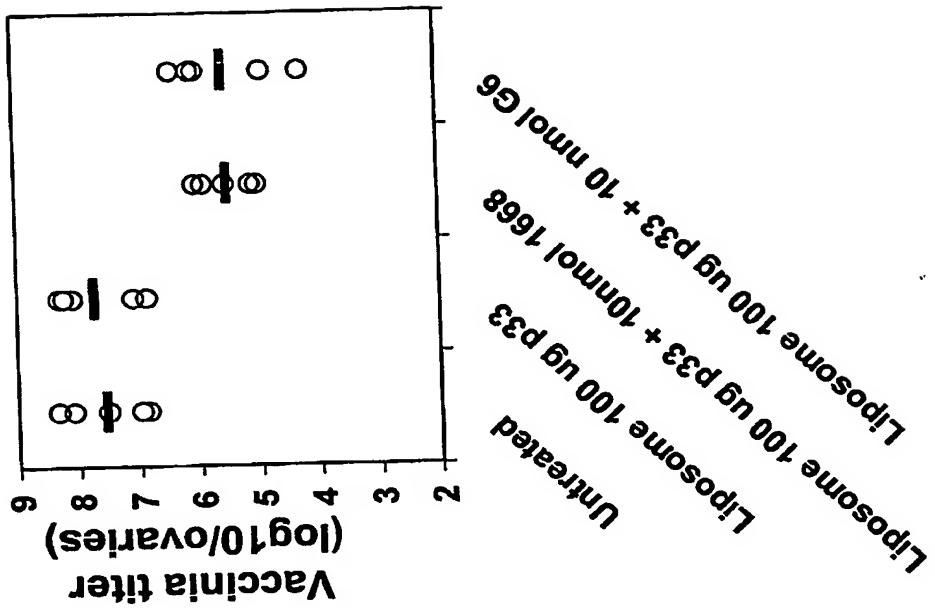
**Fig. 6**

**Fig. 7B**

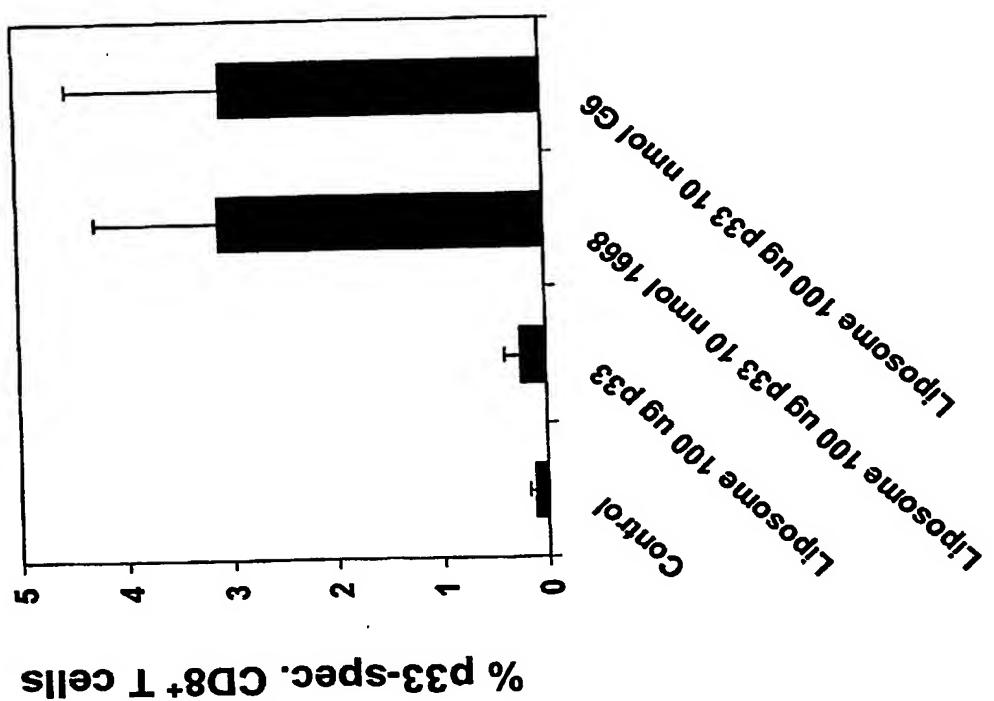


**Fig. 7A**





8B  
Fig.



**Fig. 8A**

-1-

SEQUENCE LISTING

<110> Bachmann, Martin F  
Manolova, Vania  
Storni, Tazio

<120> Enhancement of A-Type CpG-Induced IFNalpha-Production by Liposomes:  
Method of Preparation and Use

<130> 1700.0450000

<160> 13

<170> PatentIn version 3.2

<210> 1  
<211> 21  
<212> DNA  
<213> Artificial Sequence

<220>  
<221> misc\_feature  
<222> (1)..(21)  
<223> deoxynucleotides connected via phosphorothioate bonds

<400> 1  
tccatgacgt tcctgaataa t 21

<210> 2  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<221> CpG-2006

<220>  
<221> misc\_feature  
<222> (1)..(23)  
<223> deoxynucleotides connected via phosphorothioate bonds

<400> 2  
tcgtcgaaaa gtcgttttgt cgt 23

<210> 3  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<221> G10 oligonucleotide

<400> 3  
gggggggggg gacgatcgtc ggggggggggg 30

<210> 4  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> G10-PS oligonucleotide

<220>  
<221> misc\_feature  
<222> (1)..(30)  
<223> deoxynucleotides connected via phosphorothioate bonds

<400> 4 30  
gggggggggg gacgatcgac ggggggggggg

<210> 5  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> G6 oligonucleotide

<400> 5 30  
ggggggcgcac gacgatcgac gtcgggggg

<210> 6  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> G3-6 oligonucleotide

<400> 6 19  
ggggacgatc gtcgggggg

<210> 7  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> G4-6 oligonucleotide

<400> 7 20  
gggggacgat cgtcggggg

<210> 8  
<211> 21  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> G5-6 oligonucleotide

<400> 8	21
gggggggacga tcgtcgaaaa g	
<210> 9	
<211> 22	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> G6-6 oligonucleotide	
<400> 9	22
gggggggacg atcgtcgaaa gg	
<210> 10	
<211> 24	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> G7-7 oligonucleotide	
<400> 10	24
ggggggggacgatcgtcgaa gggg	
<210> 11	
<211> 26	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> G8-8 oligonucleotide	
<400> 11	26
ggggggggggca cgatcgtcgaa gggggg	
<210> 12	
<211> 28	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> G9-9 oligonucleotide	
<400> 12	28
ggggggggggca acgatcgtcgaa gggggggg	
<210> 13	
<211> 9	
<212> PRT	
<213> Artificial Sequence	
<220>	
<223> p33 peptide	
<400> 13	

-4-

Lys Ala Val Tyr Asn Phe Ala Thr Met  
1 5